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PROVISIONAL SPECIFICATION FOR AN INVENTION ENTITLED

Invention title: NON-HAEMOPOIETIC PRECURSOR CELLS

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The invention is described in the following statement:

BACKGROUND OF THE INVENTION

This invention relates to non-haemopoietic precursor cells, and the isolation of a subpopulation of such precursors by reason of the use of a perivascular marker.

Numerous attempts at isolating non-haemopoietic precursor cells have been attempted because of the potential that these cells have for medicinal use. A more recent example of such a method providing for a relatively high yield is disclosed in publication WO01/04268 to Simmons *et al.*,. The later method is admitted still to resulting in a somewhat mixed population.

The present invention relates to the finding that a population of precursor cells is present in a perivascular niche. These precursor cells can be isolated by enriching for an early perivascular surface marker, in particular the presence of CD146 and preferably additionally high level expression of the marker recognised by the monoclonal antibody STRO-1.

The identity of these precursor cells is at present not fully confirmed but might be mesenchymal or smooth muscle, in addition a subpopulation of the so-isolated precursors express the 3G5 marker and is likely to be highly enriched for pericytes. The stem cell population might be one or alternatively two or three these precursor types, and perhaps in a relatively plastic form capable of interconverting between the two or more types.

The tissues from which these cells have been isolated in the exemplifications of this invention are human bone marrow and dental pulp cells. The enriched stem cell populations have been harvested and then re-introduced to produce bone and bone marrow and dentin and pulp respectively.

It is anticipated that these precursor cell can be harvested from other tissues and may be useful for regenerating tissue for those cells types. Example of the tissue types in addition to the above might include, liver, kidney, heart, retina, brain, skin, hair follicles, intestine, lung, lymph node, thymus and pancreas. Thus precursor cells isolated from heart may be reintroduced to regenerate heart tissue, however the invention need not be so limited,

precursor cells isolated from one tissue type might be useful for regenerating tissue in another tissue type. The microenvironment in which an undifferentiated cell finds itself is known to exert an influence on the route of differentiation and therefore the reintroduction need not necessarily be tissue specific.

It will be appreciated that the precursor cells are early cells that are substantially at a pre-expansion stage of development. These are cells that have yet to differentiate to fully committed cells, however they need not be stem cells in a strict sense, in that they are necessarily able to differentiate into all types of cells. Partially differentiated precursor cells have a benefit in that they have a greater proliferative potential than fully differentiated cells.

It will thus be understood that the invention in a first aspect might be said to reside in a method of enriching precursor cells from a population containing perivascular cells including the steps of enriching for cells based on the presence of an early perivascular marker, which might be CD146, said cells also preferably being positive for α smooth muscle actin.

It is preferred that the method includes additionally enriching based on STRO-1 bright. This is a characteristic that reflects high levels of expression of the antigen recognised by the antibody STRO-1.

An alternative form of the first aspect of the present invention includes additionally enriching based on presence of the marker 3G5. Most preferably this aspect provides for enrichment based on all three of presence of CD146, 3G5 and STRO-1 bright. It is believed that the use of these three markers provides for enrichment of pericytes, and that this is the first time that that precursor cell type has been enriched to significant numbers, and in particular human pericytes. In preference this alternative form of the invention relates to the isolation of an unexpanded population of human pericytes.

Earlier attempts at isolating pericytes have largely been physical. Thus pericytes have been isolated from bovine retinas, by enzymic degradation followed by Percoll density gradient separation. The resultant cells are a mixture of perivascular and endothelial cells. The present invention provides for pericytes substantially free of endothelial cells. In that context

substantially free might be considered to be less than about 5, 2, 1, or 0.1% endothelial cells. Alternatively the context might be an assessment that the enriched population is von Willebrand Factor negative.

The specificity of the markers used in this process is not absolute. Thus even the most preferred markers occur on cell types other the precursors cells, however their expression in the combinations referred to above in the cell surfaces of other cell types is limited.

It will be understood that recognition of cells carrying the cell surface markers that form the basis of the separation can be effected by a number of different methods, however, all of these methods rely upon binding a binding agent to the marker concerned followed by a separation of those that exhibit binding, being either high level binding, or low level binding or no binding. The most convenient binding agents are antibodies or antibody based molecules, preferably being monoclonal antibodies or based on monoclonal antibodies because of the specificity of these latter agents. Antibodies can be used for both steps, however other agents might also be used, thus ligands for these markers may also be employed to enrich for cells carrying them, or lacking them.

The antibodies may be attached to a solid support to allow for a crude separation. The separation techniques should maximise the retention of viability of the fraction to be collected. Various techniques of different efficacy may be employed to obtain relatively crude separations. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill. Procedures for separation may include, but are not limited to, magnetic separation, using antibody-coated magnetic beads, affinity chromatography and "panning" with antibody attached to a solid matrix. Techniques providing accurate separation include but are not limited to FACS.

The method might include the step of making a first partially enriched pool of cells by enriching for the expression of a first of the markers, and then the step of enriching for expression of the second of the markers from the partially enriched pool of cells.

It is preferred that the method comprises a first step being a solid phase sorting step, based on recognition of one or more of the markers. The solid phase sorting step of the illustrated embodiment utilises MACS recognising high level expression of STRO-1. This then gives an

enriched pool with greater numbers of cells than if a high accuracy sort was used as a first step. If for example FACS is used first, many of the precursor cells are rejected because of their association with other cells. A second sorting step can then follow using an accurate separation method. This second sorting step might involve the use of two or more markers. Thus in the illustrated embodiment two colour FACS is used to recognise high level expression of the antigen recognised by STRO-1 as wells as the expression of CD146. The windows used for sorting in the second step can be more advantageously adjusted because the starting population is already partially enriched.

The method might also include the harvesting of a source of the stem cells before the first enrichment step using known techniques.

The preferred source of such cells is human, however, it is expected that the invention is also applicable to animals, and these might include domestic animals or animals that might be used for sport.

It will be understood that a second aspect of the present invention relates to an population or precursor cells enriched by the first aspect of the invention.

In another form the invention might be said to reside in a population of cells enriched for precursors cells exhibiting the markers CD146, STRO-1 bright, and α smooth muscle actin positive. Preferably the population is also 3G5 positive.

Preferably the population is comprised of cells that is more than about 1% , 5%, 10% or 15% colony forming.

In a further form the invention might be said to reside a method of generation tissue in a mammal comprising the step of enriching a population of precursor cells as in the first aspect of the invention, and introducing the enriched population into the mammal, and allowing the enriched population to generate the tissue in the mammal.

Another potential use for purified cells of the present invention is as a means of gene therapy, by the introduction of exogenous nucleic acids for expression of therapeutic substances in the tissue types concerned.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Properties of STRO-1$^+$ MACS-isolated cells co-labeled with anti-CD146 (CC9). (A) Sort region, R1, represents the double positive STRO-1^{BRT}/CD146$^+$ population. (B) The incidence of clonogenic cell colonies (>50 cells) based on STRO-1^{BRT}/CD146$^+$ expression was determined by limiting dilution analysis of 24 replicates per cell concentration using Poisson distribution analysis from 5 independent experiments. Forward (size) and perpendicular (granularity) light scatter characteristics of BMMNCs (C), STRO-1^{int}/CD146$^-$ cells (D) and STRO-1^{BRT}/CD146$^+$ cells (E). (F) RT-PCR analysis of STRO-1^{BRT}/CD146$^+$ sorted marrow cells for CBFA1 (lane 2), osteocalcin (lane 4) and GAPDH (lane 6) transcripts. Control cells (BMSSC cultures grown in the presence of dexamethasone) expressing CBFA1 (lane 1), osteocalcin (lane3), and GAPDH (lane 5) is also shown. Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide staining. (G) *In situ* expression of CD146 on blood vessel (bv) walls (arrow) in human bone marrow (bm) sections near the bone (b) surface 20X. Sections were counter stained with Hematoxylin. (H) Dual Immunofluorescence staining demonstrating reactivity of the STRO-1 antibody labeled with Texas red and the CC9 antibody labeled with fluorescein isothiosyanate, reacting to blood vessel walls in frozen sections of human bone marrow.

Figure 2. Immunophenotypic analysis of DPSCs *in vivo*. The bar graph depicts the number of clonogenic colonies retrieved from single cell suspensions of dental pulp following immunomagnetic bead selection based on reactivity to antibodies that recognize STRO-1, CD146, and 3G5 and isotype-matched negative control antibodies. The data are expressed as the number of colony-forming units obtained in the bead positive cell fractions as a percentage of the total number of colonies in unfractionated pulp cells averaged from three separate experiments. Statistical significance (*) was determined using the student t-test (p 0.01) comparing the percent total number of colonies for each antibody with the corresponding isotype-matched control.

Figure 3. Reactivity of perivascular makers in dental pulp. (A) Immunolocalization of the STRO-1 antigen on blood vessels (small arrows) in human dental pulp (p) and around perineurium (large arrow) surrounding a nerve bundle (nb) 20X. (B) Dual Immunofluorescence staining demonstrating reactivity of the STRO-1 antibody labeled with Texas Red to dental pulp perineurium (arrow) in combination with an anti-neurofilament antibody labeled with fluorescein isothiosyanate staining the inner nerve bundle (nb), 40X. (C) Immunolocalization of the CD146 antigen to blood vessel walls in human dental pulp tissue 20X. (D) Dual Immunofluorescence staining demonstrating reactivity of the STRO-1 antibody labeled with Texas red to a blood vessel and the CC9 antibody labeled with fluorescein isothiosyanate. (E) Immunohistochemical staining of pulp tissue with a rabbit polyclonal anti-DSP antibody (arrow) to the odontoblast outer layer (od). 20X. (F) 3G5 reactivity to a single pericyte (arrow) in a blood vessel (bv) wall 40X. Tissue sections were counter stained with Hematoxylin.

Figure 4. 3G5 reactivity to BMSSCs. (A) The representative histogram depicts a typical dual-color FACS analysis profile of whole bone marrow mononuclear cells (BMMNCs) expressing CD146 (PE) and 3G5 (FITC). (B) Colony efficiency assays were performed for all the different expression patterns observed (regions "R" 1-6). The data are expressed as the mean incidence of colony-forming units for each cell fraction averaged from three separate experiments.

Figure 5. Developmental potential of purified BMSSCs and DPSCs in vivo. Cytospin preparations of MACS/FACS isolated STRO-1^{BRT}/$CD146^{+}$ marrow cells (arrow) stained with an antibody specific to α -smooth muscle actin (A) and von Willebrand Factor (B). $CD146^{+}$ pulp cells (large arrow) isolated by immunomagnetic bead selection (magnetic beads depicted by small arrows), stained with an antibody specific to α -smooth muscle actin (C) and von Willebrand Factor. (D). (E) Ectopic bone formation (b) and haematopoietic/adipogenic marrow (bm) by *ex vivo* expanded cells derived

from STRO-1^{BRT}/CD146^{+} BMSSCs transplanted with HA/TCP into immunocompromised mice for three months (E). (F) Ectopic formation of dentin (d) and fibrous pulp tissue (p) by *ex vivo* expanded cells derived from CD146^{+} DPSCs transplanted with HA/TCP into immunocompromised mice for three months. Sections were stained with Hematoxylin & Eosin.

DETAILED DESCRIPTION OF THE ILLUSTRATED AND EXEMPLIED EMBODIMENTS OF THE INVENTION

Stem cell niches identified in a number of different adult tissues including skin, hair follicles, bone marrow, intestine, brain, pancreas and more recently dental pulp, are often highly vascularized sites.[1] The maintenance and regulation of normally quiescent stem cell populations is tightly controlled by the local microenvironment according to the requirements of the host tissue.[2,3] Both the supportive connective tissues of bone marrow and dental pulp contain stromal stem cell populations with high proliferative potentials capable of regenerating their respective microenvironments with remarkable fidelity, including the surrounding mineralized structures of bone and dentin.[4-5] In the postnatal organism, bone marrow stroma exists as a loosely woven, highly vascularized tissue that supports and regulates hematopoiesis.[6-8] At a time when many tissues have lost or decreased their ability to regenerate, adult bone marrow retains a capacity for continuous renewal of haematopoietic parenchymal tissue and is responsible for remodeling the adjoining bone surfaces.[9,10] In contrast, the inner pulp chamber of teeth is comprised of a non-hematopoietic, compact fibrous tissue, infiltrated by a microvascular network, that is entombed by mineralized dentin.[11-13] Following tooth maturation, dental pulp becomes relatively static, acting only in a reparative capacity in response to a compromised dentin matrix caused by insults such as caries or mechanical trauma.

Precursors of functional osteoblasts (BMSSCs: bone marrow stromal stem cells) and odontoblasts (DPSCs: dental pulp stem cells) were initially identified by their capacity to form clonogenic cell clusters (CFU-F: colony-forming units-fibroblast) in vitro, a common feature amongst different stem cell populations.[4,14-18] The progeny of *ex vivo* expanded BMSSCs and DPSCs share a similar gene expression profile for a variety of transcriptional regulators, extracellular matrix proteins, growth factors/receptors, cell adhesion molecules, and some, but

not all lineage markers characteristic of fibroblasts, endothelial cells, smooth muscle cells and osteoblasts.[4,19] However, previous studies have documented that individual BMSSC colonies demonstrate marked differences in their proliferation rates *in vitro* and developmental potentials *in vivo*.[5,14,20] Similar to these findings, we have recently observed comparable levels of heterogeneity in the growth and developmental capacity of different DPSC colonies.[21] Together, these studies infer a hierarchical arrangement of stromal precursor cells residing in bone marrow and dental pulp, headed by a minor population of highly proliferative pluri-potential stem cells that give rise to committed bi- and uni-potential progenitor cell populations.[22]

Despite our extensive knowledge about the properties of cultured BMSSCs and DPSCs, we still do not know if their *in vitro* characteristics are an accurate portrait of their true gene expression patterns and developmental potentials *in situ*. In addition, it is not formally known if all of the colony-forming cells within each tissue are derived from one pluri-potent stem cell pool or whether they arise from committed progenitors belonging to distinct lineages. There is also a lack of information regarding the precise anatomical location of BMSSCs and DPSCs in their respective tissues. This is mainly attributed to the rarity of stem cells and the absence of specific markers that identify different developmental stages during osteogenesis and odontogenesis, particularly for primitive subpopulations. It has previously been hypothesized that one possible niche for precursors of osteoblasts and odontoblasts may be the microvasculature networks of bone marrow and dental pulp, respectively.[23,24]

EXAMPLE 1 Isolation and expansion of precursor cells

MATERIALS AND METHODS

Tissue Samples

Bone marrow mononuclear cells (BMMNCs), from normal human adult volunteers were purchased from Poietic Technologies, Gaithersburg, Maryland. Normal human impacted third molars were collected from young adults at the Dental Clinic of the National Institute of Dental & Craniofacial Research under approved guidelines set by the NIH Office of Human Subjects Research. The pulp tissue was separated from the crown and root and then digested in a solution of collagenase type I and dispase as previously described.[4] Single cell

suspensions (0.01 to 1 x 10^5/well) of bone marrow and dental pulp were cultured in 6-well plates (Costar, Cambridge, MA) as previously described.[4] To assess colony-forming efficiency, day 14 cultures were fixed with 4% formalin, and then stained with 0.1% toluidine blue. Aggregates of ≥50 cells were scored as colonies. Paraffin and frozen human tissue sections were obtained from the Cooperative Human Tissue Network, National Cancer Institute, NIH, MD.

Magnetic-Activated Cell Sorting (MACS)

This procedure is a modification of that described elsewhere.[25] Briefly, approximately 1 x 10^8 BMMNCs were incubated with STRO-1 supernatant (murine anti-human BMSSCs, IgM)[29] (1/2) for 1 hour on ice. The cells were then washed with PBS/5% FBS and resuspended in a 1/50 dilution of biotinylated goat anti-mouse IgM (μ-chain specific; Caltag Laboratories, Burlingame, CA) for 45 minutes on ice. After washing, the cells were incubated with streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, F.R.G.) for 15 minutes on ice, then separated on a Mini MACS magnetic column (Miltenyi Biotec) according to the manufacturers recommendations.

Fluorescence activated Cell Sorting (FACS)

STRO-1-positive MACS isolated cells were incubated with a streptavidin-FITC conjugate (1/50; CALTAG Laboratories) for 20 minutes on ice then washed with PBS/5% FBS. Single-color fluorescence activated cell sorting (FACS) was performed using a FACStar^{PLUS} flow cytometer (Becton Dickinson, Sunnyvale,CA). Dual color-FACS analysis was achieved by incubating MACS-isolated STRO-1$^+$ BMMNCs with saturating (1:1) levels of CC9 antibody supernatant (mouse anti-human CD146/MUC-18/Mel-CAM, IgG_{2a}, Dr. Stan Gronthos) for one hour on ice. After washing with PBS/5% FBS, the cells were incubated with a second label goat anti-mouse IgG_2a (γ-chain specific) phycoerythrin (PE) conjugate antibody (1/50, CALTAG Laboratories) for 20 minutes on ice. The cells were then sorted using the automated cell deposition unit (ACDU) of a FACStar^{PLUS} flow cytometer. Limiting dilution assay: seeded 1, 2, 3 4, 5, & 10 cells per well, 24 replicates, cultured in serum-deprived medium for 10 days as previously described [26]. Similarly, freshly prepared unfractionated BMMNCs were incubated with CC9 (IgG_{2a}) and 3G5 (IgM) antibodies or isotype-matched negative control antibodies for one hour on ice. After washing with PBS/5% FBS, the cells were

incubated with a second label goat anti-mouse IgG_{2a} (γ-chain specific) phycoerythrin (PE) and IgM (1/50; CALTAG Laboratories) conjugated antibodies for 30 minutes on ice. Cells were washed in PBS/%5 FBS prior to being analysed using a FACStar^{PLUS} flow cytometer. Positive reactivity for each antibody was defined as the level of fluorescence greater than 99% of the isotype matched control antibodies.

Immunhistochemistry

Human tissue sections (μm) were de-waxed in xylene and rehydrated through graded ethanol into PBS. Frozen tissue sections (μm) and cytospin preparations were fixed with cold acetone at -20°C for 15 minutes then washed in PBS. The samples were subsequently treated with PBS containing 1.5% of hydrogen peroxide for 30 minutes, washed then blocked with 5% non-immune goat serum for 1 hour at room temperature. Samples were incubated with primary antibodies for 1 hour at room temperature. Antibodies used: Mouse (IgG_1 & IgG_{2a}) controls (Caltag, Burlingame, CA); Rabbit (Ig) control, 1A4 (anti-α smooth muscle actin, IgG_1), 2F11 (anti-neurofilament, IgG_1), F8/86 (murine anti-von Willebrand Factor; IgG_1) (Dako, Carpinteria, CA); STRO-1; CC9 (anti-CD146); LF-151 (rabbit anti-human dentinsialoprotein; Dr. L. Fisher, NIDCR/NIH, MD). Working dilutions: rabbit serum (1/500), monoclonal supernatants (1/2) and purified antibodies (10 μg/ml). Single staining was performed by incubating the samples with the appropriate secondary antibody, biotinylated goat anti-mouse IgM, IgG_1, IgG_{2a} or biotinylated goat anti-rabbit for one hour at room temperature (Caltag Laboratories). Avidin-Peroxidase-complex and substrate were then added according to the manufacturer instructions (Vectastain ABC Kit standard, Vector Laboratories). Samples were counterstained with hematoxylin and mounted in aqueous media. Dual-fluorescence labeling was achieved by adding the secondary antibodies, goat anti-mouse IgM-Texas Red
and IgG-FITC (CALTAG Laboratories), for 45 minutes at room temperature. After washing the samples were mounted in VECTASHIELD fluorescence mountant.

Immunomagnetic bead selection

Single cell suspensions of dental pulp tissue were incubated with antibodies reactive to STRO-1 (1/2), CD146 (1/2), or 3G5 (1/2) for 1 hour on ice. The cells were washed twice with PBS/1%BSA then incubated with either sheep anti-mouse IgG-conjugated or rat anti-mouse

IgM-congugated magnetic Dynabeads (4 beads per cell: Dynal, Oslo, Norway) for 40 minutes on a rotary mixer at 4°C. Cells binding to beads were removed using the MPC-1 magnetic particle concentrator (Dynal) following the manufactures recommended protocol.

In vivo transplantation studies

Approximately 5.0×10^6 of *ex vivo* expanded cells derived from either STRO-1^{BRT}/$CD146^+$ BMSSCs or $CD146^+$ DPSCs were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer Inc, Warsaw, IN) and then transplanted subcutaneously into the dorsal surface of 10-week-old immunocompromised beige mice (NIH-bg-nu-xid, Harlan Sprague Dawley, Indianapolis, IN) as previously described.[4] These procedures were performed in accordance to specifications of an approved animal protocol (NIDCR #00-113).

Reverse transcription-polymerase chain reaction.

Total RNA was prepared from STRO-1^{BRT}/$CD146^+$ sorted BMMNCs, and control cells (primary BMSSC cultures grown in the presence of 10^{-7} M dexamethasone for three weeks) using RNA STAT-60 (TEL-TEST Inc. Friendswood TX). First-strand cDNA synthesis was performed with a first-strand cDNA synthesis kit (GIBCO BRL, Life Technologies) using an oligo-dT primer. First strand cDNA (2 µl) was added to 46 µl of a 1X PCR master reaction mix (Roche Diagnostics, Gmbh Mannheim Germany) and 10 pMol of each human specific primer sets: CBFA1 (632bp, and three smaller alternative splice variants)[27] sense 5'-CTATGGAGAGGACGCCACGCCTGG-3', antisense, 5'-CATAGCCATCGTAGCCTTGTCCT-3'; osteocalcin (310bp)[4] sense, 5'-CATGAGAGCCCTCACA-3', antisence, 5'-AGAGCGACACCCTAGAC-3'; GAPDH (800bp) [4] sense, 5'-AGCCGCATCTTCTTTTGCGTC-3'; antisense 5'-TCATATTTGGCAGGTTTTTCT-3'. The reactions were incubated in a PCR Express Hybaid thermal cycler (Hybaid, Franklin, MA) at 95°C for 2 minutes for 1 cycle then 94°C/(30 sec), 60°C/(30 sec), 72°C/(45 sec) for 35 cycles, with a final 7 minute extension at 72°C. Following amplification, each reaction was analyzed by 1.5% agarose gel electrophoresis, and visualized by ethidium bromide staining.

RESULTS

BMSSCs and DPSCs express vascular associated antigens STRO-1 and CD146 in vivo.

We have previously demonstrated the efficacy of magnetic activated cell sorting (MACS), to isolate and enrich for all detectable clonogenic CFU-F from aspirates of human marrow, based on their high expression of STRO-1 antigen.[25,26] To further characterize BMSSCs we incubated the STRO-1$^+$ MACS isolated cells with another monoclonal antibody, CC9,[28] that recognizes the cell surface antigen CD146, also known as MUC-18, Mel-CAM and Sendo-1, that is present on endothelial and smooth muscle cells. These studies determined that CC9, selectively bound the STRO-1 bright expressing fraction (STRO-1^{BRT}) from the total STRO-1$^+$ population by dual-color FACS analysis (Figure 1A). Cloning efficiency assays using Poisson distribution statistics, yielded a marked increase in the incidence of BMSSCs (1 CFU-F per 5 STRO-1^{BRT}/CD146$^+$ cells plated), and achieved a 2 x 10^3 fold enrichment of the CFU-F population when compared to unfractionated marrow (Figure 1B). No colony formation could be detected in STRO-1^{BRT}/CD146$^-$ cell fraction (data not shown).

The light scatter properties of STRO-1^{BRT}/CD146$^+$ marrow cells were typically larger and more granular than the nucleated erythroid cells and B-lymphocytes comprising the bulk of the STRO-1$^+$ population[29] (Figure 1C-E). Cytospin preparations of STRO-1^{BRT}/CD146$^+$ sorted cells were found to be negative for the erythroid (glycophorin-A) and leukocyte (CD45) associated markers (data not shown). Confirmation that BMSSCs represented an early osteogenic precursor population was obtained by RT-PCR analysis of highly purified MACS/FACS-isolated STRO-1^{BRT}/CD146$^+$ cells, which failed to detect the early and late osteogenic, markers CBFA1 and osteocalcin, respectively (Figure 1F). However, the progeny of STRO-1^{BRT}/CD146$^+$ sorted BMSSCs were found to express both CBFA1 and osteocalcin, following *ex vivo* expansion. Immunolocalization studies demonstrated that the CD146 antigen was predominantly expressed on blood vessel walls in sections of human bone marrow (Figure 1G). Localization of both STRO-1 and CD146 was confined to large blood vessels in frozen sections of human bone marrow trephine (Figure 1H).

Immunoselection protocols were subsequently used to determine if human DPSCs also expressed STRO-1 and CD146 *in situ*. The use of either MACS or FACS analysis to isolate DPSCs was restrictive due to the rarity of these cells (1 colony-forming cell per 2 x 10^3 cells plated) compounded by the limited number of pulp cells (approximately 10^5 cells per pulp sample) obtained following processing. To circumvent this, we pooled several pulp tissues

obtained from 3 to 4 different third molars per experiment and employed immunomagnetic bead selection on single cell suspensions of pulp tissue, based on their expression of either the STRO-1 or CD146 antigens. The STRO-1$^+$ fraction represented approximately 6% of the total pulp cell population. Comparative studies demonstrated that growth rates of individual colonies were unperturbed in the presence of magnetic beads (data not shown). Colony efficiency assays indicated that the majority of dental pulp derived colony-forming cells (82%) were represented in the minor, STRO-1$^+$ cell fraction analogous to BMSSCs (Figure 2). The mean incidence of DPSCs in the STRO-1 positive fraction (329 colony-forming cells per 10^5 cells plated ± 56 SE, n=3) was six-fold greater than unfractionated pulp cells (55 colony-forming cells per 10^5 cells plated ± 14 SE, n=3). Using a similar strategy, different fractions of human dental pulp cells were selected based on their reactivity with the antibody, CC9. Colony efficiency assays showed that a high proportion (96%) of dental pulp-derived CFU-F were also present in the CD146$^+$ population, using immunomagnetic Dynal bead selection (Figure 2). The mean incidence of CFU-F in the CD146$^+$ fraction (296 colony-forming cells per 10^5 cells plated ± 37 SE, n=3) was seven-fold greater than unfractionated pulp cells (42 colony-forming cells per 10^5 cells plated ± 9 SE, n=3).

Immunolocalization studies showed that STRO-1 expression was restricted to blood vessel walls and perineurium surrounding the nerve bundles, but was not present in the mature odontoblast layer or fibrous tissue, in frozen sections of human dental pulp tissue (Figure 3A-B). Furthermore, co-localization of CD146 with STRO-1 was detected on the outer blood vessel cell walls, with no reactivity to the surrounding fibrous tissue, odontoblast layer, and the perineurium of the nerve (Figure 3C-D). Importantly, expression of human odontoblast-specific differentiation marker, dentinsialoprotein (DSP), was restricted to the outer pulpal layer containing mature odontoblasts (Figure 3E) and was absent in fibrous tissue, nerve bundles and blood vessels.

Differential expression of the perivascular marker 3G5 by BMSSCs and DPSCs.

Previous reports have determined that pericytes isolated from different anatomical sites have the capacity to differentiate into functional osteoblasts and other stromal cell types both *in vitro* and *in vivo*.[30] In the present study, flow cytometric analysis revealed that the pericyte-associated cell surface antigen, 3G5, was highly expressed by a large proportion (54%) of

hematopoietic marrow cells (Figure 4A). This observation eliminated 3G5 as a candidate marker for isolating purified populations of BMSSCs directly from aspirates of human marrow. In addition, dual-FACS analysis based on 3G5 and STRO-1 expression was not possible since both antibodies shared the same isotype. Nevertheless, *in vitro* colony efficiency assays for different 3G5/CD146 FACS sorted subfractions demonstrated that only a minor proportion (14%) of bone marrow CFU-F expressed the 3G5 antigen at low levels (Figure 4B). Conversely, a larger proportion (63%) of clonogenic DPSCs (192 colony-forming cells per 10^5 cells plated ± 18.4 SE n=3) were present in the $3G5^+$ cell fraction following immunomagnetic bead selection (Figure 2). Significantly, 3G5 demonstrated specific reactivity to pericytes in frozen sections of human dental pulp tissue (Figure 3F).

We next analyzed the expression of more specific markers of endothelial cells (von Willebrand Factor) and smooth muscle cells/pericytes (α -smooth muscle actin) on cytospin preparations using freshly isolated STRO-1^{BRT}/$CD146^+$ BMSSCs and $CD146^+$ expressing DPSCs. A large proportion of purified BMSSCs (67%), were found to be positive for α -smooth muscle actin (Figure 5A), but lacked expression of von Willebrand Factor (Figure 5B). Similarly, the majority of isolated DPSCs (85%) were also found to express α -smooth muscle actin, but not von Willebrand Factor (Figure 5C, 5D). Purified populations of STRO-1^{BRT}/$CD146^+$ BMSSCs and $CD146^+$ DPSCs were subsequently expanded *in vitro* then transplanted into immunocompromised mice to assess their developmental potentials *in vivo*. The progeny of cultured BMSSCs and DPSCs displayed distinct capacities, capable of regenerating the bone marrow and dental/pulp microenvironments, respectively (Figure 5E, F), and appeared identical to the developmental potential of non-selected multi-colony derived BMSSCs and DPSCs (4).

DISCUSSION

Circumstantial evidence, based on histological examination of bone marrow, suggests that precursors of osteoblasts arise from proliferating perivascular cells budding from the abluminal surfaces of marrow sinuses undergoing angiogenesis.[24,31] Similar studies have postulated that pre-odontoblasts may originate from perivascular cells migrating out of capillary walls into the surrounding fibrous pulp tissue, in response to degradation of the dentin matrix.[23,30] The present study provides direct evidence that two mesenchymal stem

cell populations, distinct in their ontogeny and developmental potentials, are both associated with the microvasculature of their respective tissues. The majority of BMSSCs were found to display characteristics of smooth muscle cells while most DPSCs presented with a phenotype consistent with pericytes.

We employed different immunoselection protocols to demonstrate that BMSSCs and DPSCs could be efficiently retrieved from bone marrow aspirates and enzyme digested pulp tissue respectively, based primarily on their high expression of the STRO-1 antigen. This cell surface antigen is present on precursors of various stromal cell types including, marrow fibroblasts, osteoblasts, chondrocytes, adipocytes, and smooth muscle cells isolated from human adult and fetal bone marrow.[29,32-34] Previous studies have implicated STRO-1 as a marker of pre-osteogenic populations, where its expression is progressively lost following cell proliferation and differentiation into mature osteoblasts in vitro.[27,35,36] The STRO-1 antigen was also found to be present on the outer cell walls of human bone marrow and dental pulp blood vessels, in accord with previous studies that localized STRO-1 on large blood vessels, but not capillaries, in different adult tissues such as brain, gut, heart, kidney, liver, lung, lymphnode, muscle, thymus.[6] Therefore, STRO-1 appears to be an early marker of different mesenchymal stem cell populations and infers a possible perivascular niche for these stem cell populations *in situ*.

To determine if BMSSCs and DPSCs were associated directly with blood vessels we utilized another antibody (CC9),[28] which recognizes the immunoglobulin super family member, CD146 (MUC-18/Mel-CAM), known to be present on smooth muscle, endothelium, myofibroblasts and Schwann cells *in situ*, as well as being a marker for some human neoplasms.[37] Notably, CD146 is not expressed by bone marrow hematopoietic stem cells, nor their progenitors. While the precise function of CD146 is not known, it has been linked to various cellular processes including cell adhesion, cytoskeletal reorganization, cell shape, migration and proliferation through transmembrane signaling.

In order to dissect the BMSSC population, STRO-1^{BRT} expressing marrow cells were further distinguished from STRO-1^{+} hematopoietic cells (predominantly glycophorin-A^{+} nucleated erythrocytes) based on their expression of CD146, using dual-FACS analysis. Purified STRO-1^{BRT}/$CD146^{+}$ human BMSSCs displayed light scatter properties characteristic of large granular

cells. Our study supports the findings of Van Vlasselaer and colleagues (1994)[38] who isolated partially purified BMSSCs from murine bone marrow following 5-fluoracil (5-FU) treatment, and identified this population as having high perpendicular and forward light scatter characteristics. Interestingly, freshly isolated 5-FU resistant murine BMSSCs were also found to be positive for two perivascular markers Sab-1 and Sab-2.[38] Conversely, more recent studies have shown that when BMSSCs are cultivated *in vitro*, the most primitive populations display low perpendicular and forward light scatter properties[39] and therefore may not reflect the true morphology of BMSSC *in situ*. In the present study, STRO-1^{BRT}/CD146^{+} sorted human BMSSCs lacked the expression of CBFA1 and osteocalcin that identify committed early and late osteogenic populations, respectively,[40,41] indicating that BMSSCs exhibit a pre-osteogenic phenotype in human bone marrow aspirates. We found that a high proportion of freshly isolated STRO-1^{BRT}/CD146^{+} BMSSCs expressed α -smooth muscle actin, but not the endothelial specific marker von Willebrand Factor, providing direct evidence that this primitive precursor population displays a characteristic perivascular phenotype.

The present study also demonstrated the efficacy of using magnetic bead selection to isolate and enrich for DPSCs directly from human dental pulp tissue based on their expression of either STRO-1 or CD146. Immunolocalization of CD146 appeared to be specific to the microvasculature within dental pup. Co-localization of both STRO-1 and CD146 on the outer walls of large blood vessel in dental pulp tissue, implied that the majority of DPSCs arise from the microvasculature. However, since the STRO-1 antibody also reacted with the perineurium in dental pulp and peripheral nerve bundles (unpublished observations), further investigation is required to determine the role of this antigen in neural cell development.

Analogous to BMSSCs, freshly isolated CD146^{+} DPSCs were found to express α -smooth muscle actin but not von Willebrand Factor. DPSCs were also shown to be an immature pre-odontogenic population both by their location distal from the dentin forming surface and by their lack of expression of the human odontoblast-specific dentin sialoprotein (DSP), which is restricted to the outer pulpal layer containing differentiated odontoblasts. We have previously described that *ex vivo* expanded human DPSCs do not express the precursor molecule, dentinsialophosphoprotein (DSPP), *in vitro* when cultured under non-inductive conditions.[4] Similar studies have shown that DSPP mRNA was highly expressed in freshly isolated

odontoblast/pulp tissue, but was not detect in cultured dental papilla cells derived from rat incisors.[43,44] It is only when DPSCs are induced, either *in vitro*,[45] or by *in vivo* transplantation to form an ordered dentin matrix that DSPP is expressed.[c]

In vitro studies of *ex vivo* expanded BMSSCs and DPSCs supported the notion that their progeny were morphologically similar to cultured perivascular cells having a bi-polar fibroblastic, stellar or flat morphology, rather than a polygonal endothelial-like appearance. In addition, we have previously shown that the progeny of BMSSC- and DPSC-derived colonies exhibit heterogeneous staining for both CD146 and α -smooth muscle actin, but lack expression of the endothelial markers, CD34 and von Willebrand Factor, *in vitro*.[4] This prompted us to investigate whether BMSSCs and DPSCs were in fact smooth muscle cells, pericytes or a composite of both lineages. Mounting evidence suggests that, in addition to participating in the maintenance of blood vessel homeostasis, pericytes may also represent multipotential mesenchymal stem cells.[23,46-48] Some studies have proposed that pericytes may be precursors of endothelial and/or smooth muscle cells, but the exact developmental relationship between all three cell lineages is obscure during angiogenesis.[30,47,49] Pericytes isolated from bovine retinal capillaries exhibit the potential to differentiate into a variety of cell types including, osteoblasts, adipocytes, chondrocytes and fibroblasts.[23,47,50] Notably, cultured bovine pericytes are STRO-1 positive[23] demonstrating the fidelity of STRO-1 as a marker of primitive mesenchymal progenitors derived from diverse tissues. However, the ability to distinguish cultured pericytes from other vascular cell types and to follow their fate *in vivo* is difficult to assess due to their common protein expression patterns and because of the absence of lineage specific makers.[47] Nevertheless, comparative immunophenotypic analysis of fibroblasts, smooth muscle cells, and endothelial cells *in vitro* has established basic profiles that can differentiate pericytes from other cellular components of blood vessels *in vitro*.[30]

One differential marker, 3G5, was initially described based on its reactivity to an o-acetylated disialoganglioside epitope on the surface of capillary perycites.[51,52] However, in the present study we found that 3G5, is also present on the majority of the bone marrow mononuclear cell population. Flow cytometric studies demonstrated a low expression of 3G5 by BMSSCs, suggesting that only a minor subset of marrow stromal progenitors were pericyte-like, as

would be expected since BMSSCs are also present distal from blood vessels as demonstrated by their expression of nerve growth factor-receptor[26] also present on marrow reticular cells *in vivo*.[5] The observation that the majority of DPSCs expressed the pericyte marker, 3G5, was significant when taken in context with the specific reactivity of 3G5 to pericytes within the microvasculature of dental pulp tissue.[31] The differential expression of 3G5 by BMSSCs and DPSCs may reflect the contrasting regenerative capacities of their respective tissues. These findings may represent a common ontogeny between dental pulp tissue and pericytes, where both are thought to originate from migratory neural crest cells during embryogenesis.[30] In contrast, the connective tissue of bone marrow forms as a consequence of invading blood vessels and associated mesodermal derived mesenchyme, penetrating into the medullary cannals of rudimentary bone, and may explain the smooth muscle-like phenotype of BMSSCs in adult marrow.[31] Our *in vivo* phenotypic analysis of BMSSCs is consistent with the findings of Chabord and colleagues[54,55] that describe BMSSCs as having a vascular smooth muscle cell-like phenotype *in vitro*.

The observations that two different mesenchymal stem cell populations such as BMSSCs and DPSCs harbour in perivascular niches may have further implications for identifying stem cell populations in other adult tissues. Recent findings have identified human "reserve" multi-potent mesenchymal stem cells in connective tissues of skeletal muscle, and dermis derived from human fetal and adult samples.[56] However the exact location, developmental potential and ontogeny of these stem cells is still largely unknown. In the present study, identification of mesenchymal stem cell niches in bone marrow and dentin pulp may help elucidate the fundamental conditions necessary to selectively maintain and expand primitive multi-potential populations *in vitro*, in order to direct their developmental potentials *in vivo*.

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Various features of the invention have been particularly shown and described in connection with the exemplified embodiments of the invention, however, it must be understood that these particular arrangements merely illustrate and that the invention is not limited thereto and can include various modifications falling within the spirit and scope of the invention.

Dated this 28th day of March 2003

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A.P.T. Patent and Trade Mark Attorneys

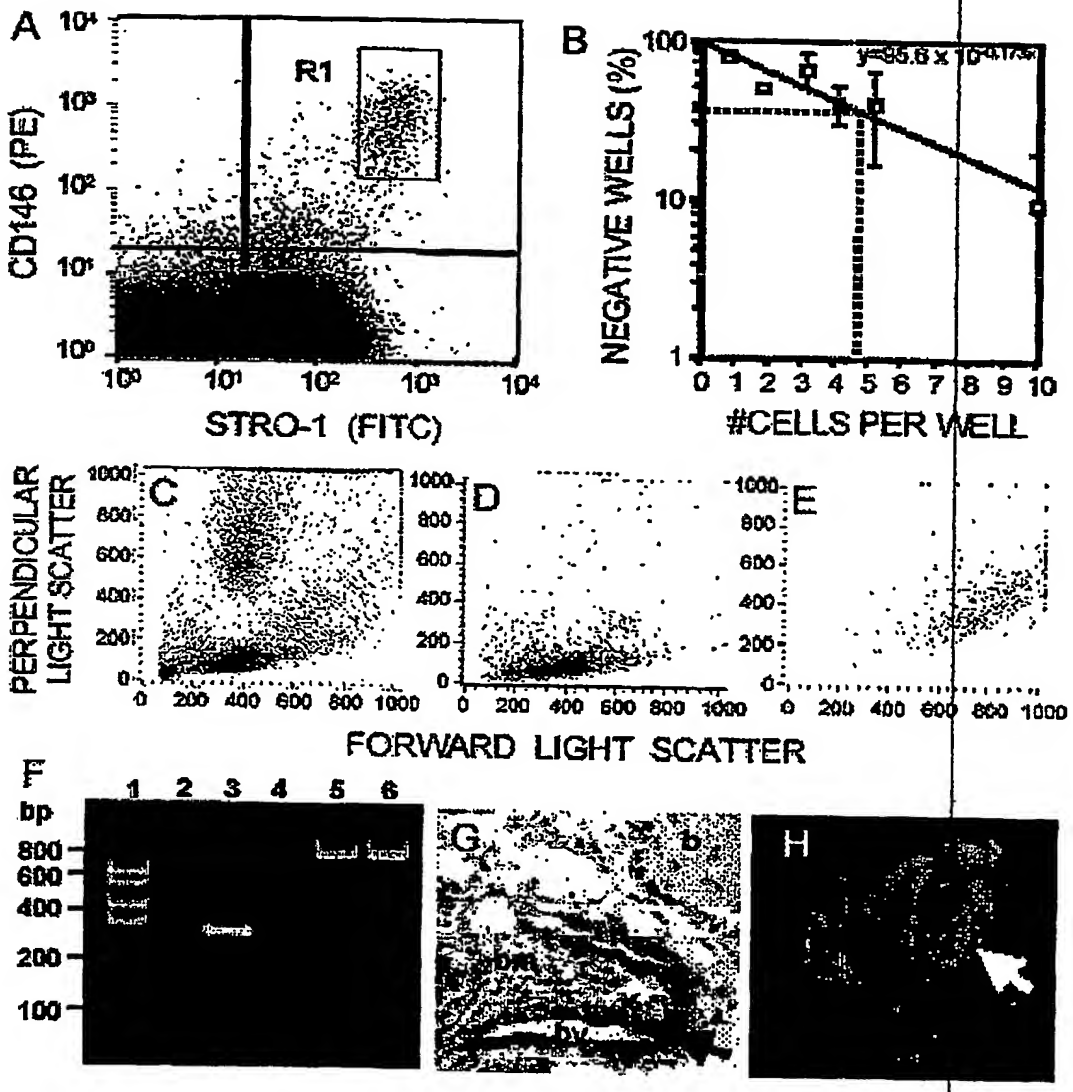

A
R1
CD146 (PE)
STRO-1 (FITC)
B
NEGATIVE WELLS (%)
#CELLS PER WELL
C
D
E
PERPENDICULAR LIGHT SCATTER
FORWARD LIGHT SCATTER
F
bp
800
600
400
200
100
G
H

FIGURE 1

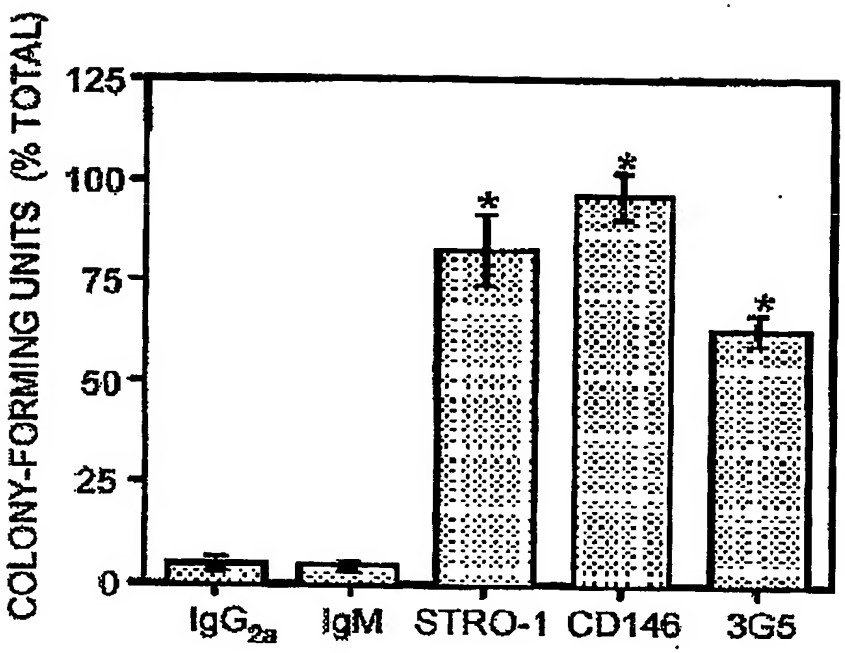

COLONY-FORMING UNITS (% TOTAL)
125
100
75
50
25
0
*
*
*
IgG2a
IgM
STRO-1
CD146
3G5

FIGURE 2

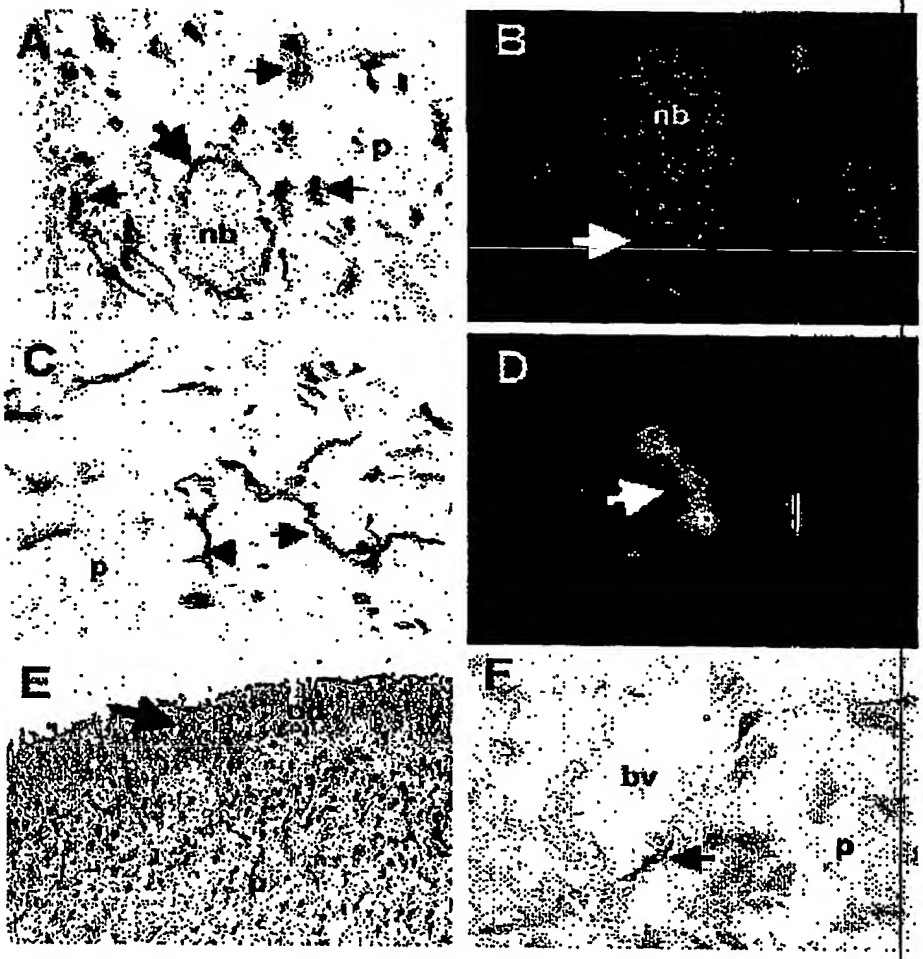
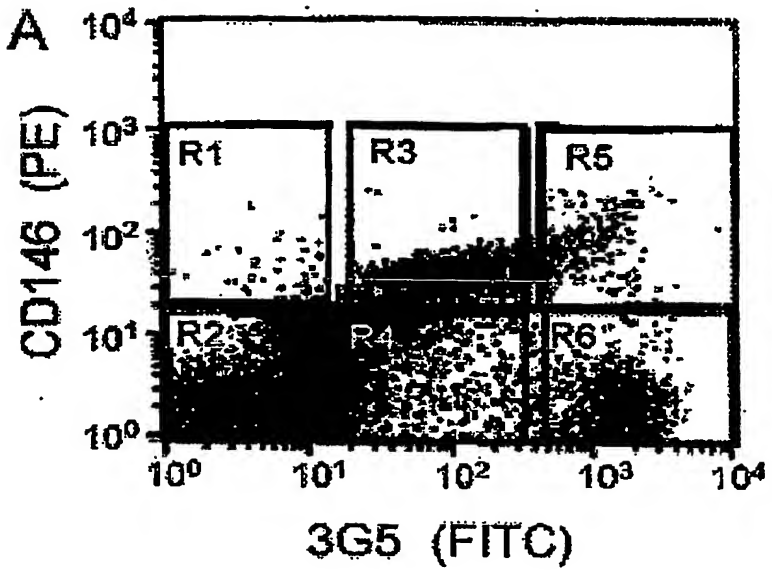


FIGURE 3

A



B

Cell Fraction	# CFU-F/10^5 cells
BMMNC	13 ± 2.6
R1: $3G5^-/CD146^+$	360 ± 45.0
R2: $3G5^-/CD146^-$	0
R3: $3G5^+/CD146^+$	58 ± 11.2
R4: $3G5^+/CD146^-$	0
R5: $3G5^{++}/CD146^+$	1 ± 1.0
R6: $3G5^{++}/CD146^-$	0

FIGURE 4

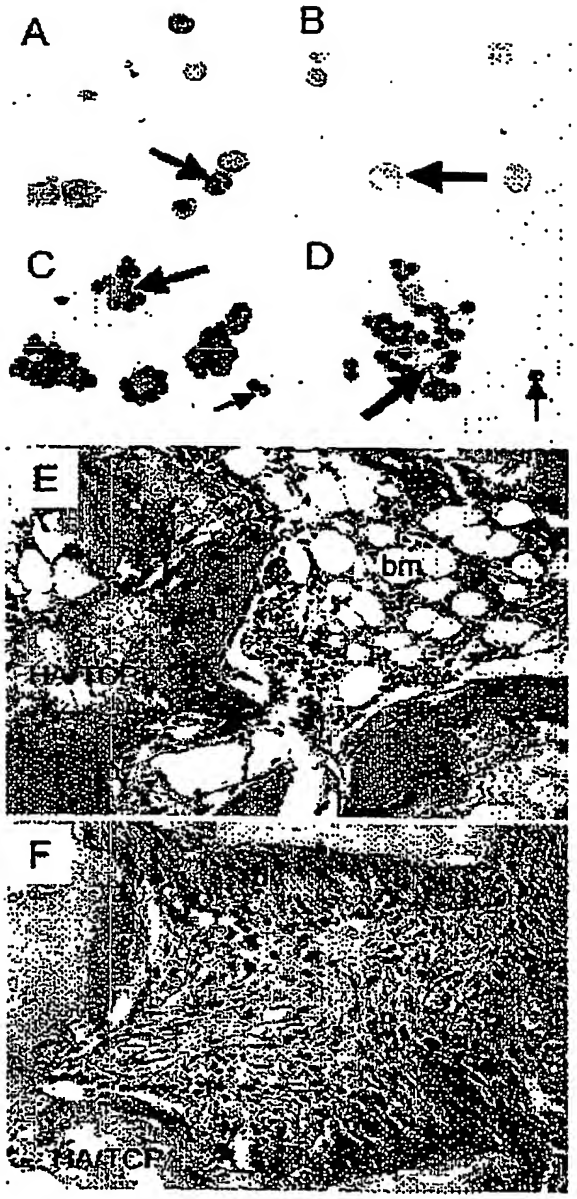


FIGURE 5

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